B cell clonality in gastric lymphoid tissues of patients with Sjögren's syndrome†

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Abstract

Objective—To determine the prevalence of mucosa associated lymphoid tissue (MALT) in the stomach and of a possible antigen driven proliferation, in patients with Sjögren's syndrome (SS).

Methods—Twenty one patients with primary SS and 80 dyspeptic controls underwent upper endoscopy. Lymphoid tissue and *Helicobacter pylori* were assessed by histopathological analysis. Epstein-Barr virus (EBV) or human herpes virus-6 (HHV-6) genome were studied by polymerase chain reaction (PCR) DNA amplification. Two PCR VDJ procedures were used to detect immunoglobulin heavy chain (IgH) gene rearrangement.

Results—Organised MALT was found in 33.3% of the patients, compared with 21.5% of the controls (NS). *H. pylori* infection was seen in 71% of patients and 63% of controls. Genomic EBV or HHV-6 was found in a minor portion of SS gastric tissues. B cell expansion was detected in nine of the 21 patients. Infectious agents in the stomach might have contributed to B cell clonality only in 55.5% of the cases. No strict relationship was found between lymphoid follicles and clonality.

Conclusion—Lymphoid accumulation in the gastric mucosa is common in Sjögren's syndrome, but full evidence for an antigen driven B cell expansion could not be demonstrated. Only a portion of those with clonal B cell expansion had evidence of an infectious agent. Other unknown infectious agents or factors related to the underlying disease (auto-antigen) and its tissue environment may have a further role as possible causes of B cell clonal expansion in the gastric mucosa.

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Isaacson's group originated the concept of lymphoproliferative diseases of the mucosa associated lymphoid tissue (MALT),¹ describing the expansion of migrated lymphoid cells to the gastric mucosa, which normally does not contain organised lymphocytes.¹ It has recently been proposed that, in the presence of a persistent antigenic stimulus, MALT may proliferate and even transform into a full blown lymphoma.²

Sjögren's syndrome (SS) is a disease characterised by lymphoid infiltration of the salivary

glands, and frequent involvement of the gastrointestinal tract. Several agents have been considered as possible chronic stimuli (for example Epstein-Barr Virus (EBV), retroviruses, human herpes virus-6 (HHV-6)), and it may be that different agents are involved in different tissues.^{3–5}

In the stomach, *Helicobacter pylori* has been claimed to be the main agent responsible for the appearance of lymphoid infiltrates.^{6–7} As SS may in itself represent a likely cause of lymphoid tissue migration to the mucosa of the gastrointestinal tract,⁸ we thought it would be of interest to study the gastric tissue in SS and define whether the presence of MALT in the stomach is a common occurrence in SS (patients with SS often report unspecific dyspeptic symptoms, attributed to a coexistent 'chronic gastritis',⁹ whether the presence of MALT in the stomach is associated with infectious agents such as *H. pylori* or viruses implicated in the pathogenesis of SS,^{3–5} and whether gastric MALT shows evidence of lymphoid proliferation and B cell clonal expansion possibly related to local chronic antigenic stimulation.^{10–11}

Patients and methods

Twenty one consecutive patients (median age 52 (range 30–70) years) among 50 with a diagnosis of primary SS according to the recently proposed criteria¹² gave their consent to enter the study. The diagnostic criteria have yet to be fully validated, but may be considered at least as suitable as any others available in the literature. The patients were studied on an outpatient basis at the Rheumatic Disease Unit of the Department of Internal Medicine. All 21 had undergone salivary gland biopsy as part of the diagnostic procedure and 16 of them were of grade 3–4 according to Chisholm and Mason;¹³ the others had lower grading, but fully satisfied the criteria of sicca syndrome (xerostomia, salivary gland hypofunction, keratoconjunctivitis sicca, positive autoantibodies).

None of the patients had been taking xerogenic drugs, steroids, antibiotics or non-steroidal anti-inflammatory drugs in the previous three months. In the past, seven of these patients had taken brief courses of amoxicillin/ampicillin because of parotid infections, and 15 had taken low doses of steroids (prednisone 5–7.5 mg/day) for short periods of time (two to eight weeks) because of arthralgia, arthritis, intercurrent swelling of their glands without infection, or other extraglandular manifestations.

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The study included a full clinical and immunological investigation: salivary gland and lachrymal function were tested, antinuclear antibodies (ANA), extractable nuclear antigen (ENA) and rheumatoid factor (RF) were measured, and HLA locus I and II typing was performed.

The gastritis was diagnosed histopathologically and its relationship with local infectious agents investigated. Evidence of B cell clonal expansion was sought in sera using a sensitive immunofixation technique able to demonstrate a monoclonal gammopathy, and tissue clonal B cell expansion was tested by genotypic analysis of the gastric tissue. The behaviour of the possible B cell clonal expansion and its relationship with *H. pylori* infection over time after treatment was investigated.

A control group of 80 patients, referred to us because of simple dyspepsia, but without any clinical sign of overt immunological diseases, underwent upper endoscopy with gastric biopsying.

HISTOPATHOLOGY

Upper endoscopy was performed using standard Olympus video instruments. Routinely, an average of five or six biopsy specimens were obtained from the antrum for histology, another was obtained for rapid urease test (CLO from Delta West, Bentley, Australia), and three more were taken for molecular studies; they were immediately frozen in liquid nitrogen and stored at -70°C . Virtually all the specimens included the muscularis mucosa. Samples from each specimen were mounted on slides and stained with haematoxylin-eosin and with a modified Giemsa stain to study the degree of inflammation and the presence of lymphoid follicles and *H. pylori*. The lymphoid component was graded on a scale of 0 (scattered plasma cells in lamina propria without lymphoid follicles) to 5 (diffuse infiltration—prominent lympho-epithelial lesions) (figure) according to the system proposed by Wotherspoon *et al.*¹⁴ A lymphoid follicle was defined as such when an unequivocal germinal centre was seen. Immunophenotype data of the lymphoid population were collected for B (CD20-L26-DAKO), T (CD3-T cell-DAKO), and κ/λ chain (DAKO) restriction. Each set of biopsy specimens was examined by two pathologists (CAB and CA) blinded as to the clinical diagnosis and treatment, and the final grading represents the average of the two individual scores, the agreement being more than 90%. Patients were classified as *H. pylori* positive or negative on the basis of both histology and the urease CLO test. When the two tests did not agree (two cases), classification was based on histology.

GENOTYPIC ANALYSIS

DNA was extracted from fresh frozen gastric specimens using standard procedures, and subjected to molecular studies after positive β globin gene amplification.^{15 16} Amplification of the IgH gene was performed in triplicate using two different seminested polymerase chain

reaction (PCR) procedures, as described previously.¹¹ Briefly, the upstream consensus primer was complementary to the third framework V region of the IgH gene in the first procedure (Fr3), and to the second framework V region in the second procedure (Fr2). The downstream primers were the same in both the procedures, and were complementary to an outer conserved sequence of the J region in the first round of amplification, and to an inner conserved J region in the second round. The PCR product was run and evaluated optically on ethidium bromide stained agarose gel. B cell clonality was disclosed by one or more dominant bands within the predicted size range, and a smear without dominant bands within the predicted size range was taken as evidence of polyclonality. Positive and negative controls (B cell chronic leukaemia, reactive lymphnode, or no DNA) were routinely analysed and compared with the test samples.¹¹ Further molecular analysis by radioactive VDJ PCR run on polyacrylamide gel and by DNA sequencing of the PCR product confirmed the validity of the procedure (manuscript in preparation).

EBV and HHV-6 sequences were sought by PCR techniques of comparable sensitivity, as described previously.¹⁵ Particular care was taken to avoid carryover artefacts, and this was obtained by analysing for EBV and HHV-6 in different laboratories (Aviano and Ferrara, respectively). Briefly, the presence of the EBV genome in tissue samples was investigated by amplification (35 rounds) of the EBV IR3 region, followed by Southern blot hybridisation of the PCR product by an internal ^{32}P -labelled IR3 specific oligoprobe. HHV-6 was sought using the sets of primers described by Aubin *et al.*¹⁷ and specific ^{32}P -labelled oligoprobe hybridisation of the PCR product.¹⁵ To increase sensitivity, the samples were also analysed by nested PCR, amplifying a 659 bp fragment internal to the first round 832 bp product.

STATISTICAL ANALYSIS

The χ^2 exact test was used to determine differences for prevalence values between groups.

Results

Our patient population with primary SS had classical clinical, immunological, and histopathological findings: sialometry < 1.5 ml/15 min; Shirmer test < 5 mm/5 min; focal sialoadenitis (grade 3–4) in the majority of the patients; 60% were DR3 positive; all were ANA (60%) or RF (60%) positive, or both. Ro antibodies were found in 42% and La antibodies in 21%. Five patients had low serum levels of monoclonal gammopathy. Table 1 summarises the overall results of the analysis of infectious agents in the tissues and the related B cell clonal expansion.

MALT TISSUE IN THE STOMACH

We chose grade 2 (see figure) as the reference grading for MALT because it is histologically

Gastric tissue lymphoid infiltrate according to Wotherspoon-Isaacson grading (WTG) score. **A:** Grade 0 = scattered plasma cells in lamina propria; no lymphoid follicles. **B:** Grade 1 = small clusters of lymphocytes in lamina propria; no lymphoid follicles; no lymphoepithelial lesions. **C:** Grade 2 = prominent lymphoid follicles with surrounding mantle zone and plasma cells; no lymphoepithelial lesions. **D:** Grade 3 = lymphoid follicles surrounded by small lymphocytes that infiltrate diffusely in lamina propria and occasionally into epithelium. **E:** Grade 4 = lymphoid follicles surrounded by centrocyte like cells that infiltrate diffusely in lamina propria (suspicious lymphoid infiltrate probably lymphoma). **F:** Grade 5 = diffuse infiltration by centrocyte like cells; prominent lymphoepithelial lesions (lymphoma).

better defined than grade 1 and more easily recognised as a screening diagnosis. An organised (grade 2 or more) lymphoid tissue (MALT) as defined by Wotherspoon *et al*,¹⁴ was found in 33.3% of patients with SS, and in 21.5% (17 of 80) of disease control subjects (table 2); the difference between the two prevalences is not significant. A grade 3 or 4 tissue was observed in 14.3% of these patients with SS. Immunohistochemistry data revealed a lymphoid component comprised of T lymphocytes in the subepithelial region, associated with the usual B phenotype in the germinal centre and in the epithelium infiltrating lymphocytes. More detailed results of the histopathological study will be reported elsewhere. *H. pylori* infection was detected in 15 of 21 samples (71%), in five of seven (71%) of those

with lymphoid follicles, but also in 10 of 14 (71%) of those without follicles.

EBV genome was detected in two of the 21 SS samples (9.5%) and, more specifically, in two of the seven samples with lymphoid follicles, but in none of those lacking them. In contrast, HHV-6 was found in three of the 21 SS samples (14.5%)—two of seven with, and one of four without lymphoid follicles. Interestingly, only one of the three patients giving HHV-6 positive samples had the genome in blood cells.

CLONAL ANALYSIS

No evidence of $\kappa\lambda$ light chain restriction was found by immunohistochemistry in any patient. Clonality was found in nine (42.8%)

Table 1 *Helicobacter pylori* infection and virological genotypic characteristics of gastric tissues in samples from patients with primary Sjögren's syndrome (VDJ clonality reported according to Fr2 or Fr3 procedure positivity)

Patient No	HP	EBV	HHV-6	Clonality by VDJ PCR
1	-	+	-	-
2	+	-	-	+
3	+	-	-	+
4	-	nt	-	+
5	+	-	-	-
6	-	-	-	+
7	+	-	-	-
8	+	-	-	-
9	+	-	-	-
10	+	+	-	-
11	+	-	+	-
12	-	-	+	+
13	-	-	-	+
14	+	-	+	-
15	+	nt	-	-
16	-	-	-	+
17	+	-	-	-
18	-	-	-	+
19	+	-	-	-
20	+	-	-	+
21	+	-	-	-

HP = *Helicobacter pylori*; EBV = Epstein-Barr virus; HHV 6 = human herpes virus 6; VDJ = Clonal rearrangement detected through the Fr2 and Fr3 procedures (see materials and methods). nt = Not tested; + = positive, - = negative response to test.

of the 21 samples processed by VDJ PCR (in five, by Fr2 and Fr3 procedures simultaneously, and in four by either the Fr2 or the Fr3 procedure). The five patients with monoclonal gammopathies in their sera were all positive for VDJ clonality in the stomach.

Among the nine SS tissues with evidence of B cell clonality, four samples had *H. pylori*, and one had HHV-6.

SECOND BIOPSY

Four patients negative for *H. pylori* underwent a second endoscopy with gastric biopsying at least three months after the previous one, together with six patients positive for *H. pylori* who were treated with a standard eradication treatment (omeprazole 40 mg daily for two weeks and 20 mg daily for four more weeks, with amoxicillin 500 mg four times daily for the first two weeks). At the control assessment, one more, initially apparently negative, patient had become positive for *H. pylori* while, of six treated, three were negative on the second biopsy. Thus after the second biopsy, of 10 patients tested, four were *H. pylori* positive, compared with the six initially positive. All those cleared of *H. pylori* had a decrease in their Wotherspoon-Isaacson grading score. The number of samples showing an increased score for lymphoid infiltration had increased from two to three (table 3).

Table 2 *Lymphoid follicles (LF) and Helicobacter pylori* (HP) infection in patients with Sjögren's syndrome and dyspeptic controls

Patients	LF(+)	HP(+)
Sjögren's (n = 21)	7 (33.3%)	15 (71%)
Controls (n = 80)	17 (21.5%)	50 (63%)
χ^2	1.29†	0.10†

†Not significant (χ^2 non-parametric test, comparing % values for patients and controls).

Table 3 *Helicobacter pylori* (HP) and VDJ analysis in tissues graded according to the Wotherspoon-Isaacson grading score: follow up data in patients with Sjögren's syndrome

Patient No	HP-1	HP-2	VDJ-1	VDJ-2	WIG-1	WIG-2
1	-	-	-	+	1	1
2	+	+	+	+	1	2
4	-	+/-	+	+	2	1
10	+	+	-	-	2	2
11	+	+	-	+	1	3
12	-	-	+	-	3/4	3
17	+	-	-	+	2	1
18	-	-	+	+	1	2/3
19	+	-	-	-	3	2
20	+	-	+	+	1	1

HP-1, 2 = *Helicobacter pylori* positive or negative in the first or second gastric biopsy; VDJ = B cell clonal analysis; WIG = Wotherspoon-Isaacson grading score for lymphoid infiltration of the gastric tissue.

RELATIONSHIP BETWEEN CLONAL B CELL EXPANSION AND MALT

As far as the relationship between VDJ clonal expansion and lymphoid follicles in the stomach is concerned, only 14% of those with such follicles (one of seven) were VDJ positive, compared with 57% (eight of 14) of those lacking lymphoid follicles (grade 1). The χ^2 value of 3.49 is barely significant for a mutual exclusion (table 4).

CLONAL EXPANSION AND INFECTION

We found that *H. pylori* infection explained only 44.4% of all VDJ clonality at the first assessment. After the second biopsy, the number of *H. pylori* positive samples had decreased, yet the number of VDJ positive samples was increased (table 3). *H. pylori* infection in these patients seems to be resistant to the common treatment strategy for its eradication (Sorrentino *et al*, manuscript in preparation): only three of six samples were cleared of the bacilli. Interestingly, one patient who was VDJ negative at the first assessment and did not receive treatment had become VDJ positive at the second biopsy and, conversely, one initially VDJ positive patient had become VDJ negative at the second sample.

Only one sample of nine with clonal expansion had a Wotherspoon-Isaacson grading score grade 3/4; the others were grade 1 or 2.

MALT IN CONTROL SUBJECTS

Organised lymphoid tissue was detected in 21.5% of the samples, and *H. pylori* infection in 63%. *H. pylori* infection was found in 82% of the gastric specimens with lymphoid follicles.

While the majority of VDJ clonal expansion in SS was observed in those without lymphoid follicles, in the controls the opposite was seen, as reported elsewhere.¹⁸

Table 4 VDJ clonality, *Helicobacter pylori* (HP), Epstein-Barr virus (EBV), and human herpes virus 6 (HHV-6) infections related to lymphoid follicles in patients with Sjögren's syndrome

Patients	VDJ(+)	HP(+)	EBV(+)	HHV-6(+)
LF positive (n = 7)	1 (14%)*	5 (71%)	2 (29%)	2 (29%)
LF negative (n = 14)	8 (57%)	10 (71%)	0	1 (7%)

LF = Lymphoid follicles. * χ^2 = 3.49: 0.05 < p < 0.1.

Discussion

Gastritis of SS was previously labelled as an atrophic gastritis with or without intestinal metaplasia.^{19, 20} In previous studies, gastric biopsy specimens have shown mononuclear cell infiltrates of the lamina propria, and the prevalent cells were typed as T lymphocytes of the CD4 phenotype;²¹ our findings confirm these data.

The immunophenotypic analysis of the lymphoid cells infiltrating the mucosa resembles very closely that found in minor salivary glands. In this study, we observed gastric lymphoid follicles in a slightly greater percentage of SS patients compared with the control group. Lymphoepithelial lesions with evidence of B lymphocytes infiltrating the epithelium, consistent with the criteria for 'suspicious lymphoid infiltrate' according to Wotherspoon *et al*,¹⁴ were found in 14.3% of the SS samples.

There is a growing consensus concerning the role of *H. pylori* in the development of lymphoid follicles in the general population; however we found no such relationship in SS.

The first important finding of this study was that abnormal accumulation of MALT may occur in the stomach even in the absence of *H. pylori* infection in Sjögren's syndrome. Lymphoid follicles were present in only 33% of the SS gastric specimens with evidence of *H. pylori* infection. This point deserves to be stressed, as an almost uniform relationship between *H. pylori* and follicles has been reported in the literature. In the study by Wotherspoon *et al*,¹⁴ *H. pylori* was found in 92% of those with gastric MALT lymphomas that were diagnosed when B cells infiltrated the epithelium to form lymphoepithelial lesions. Genta *et al*²² reported that all subjects with *H. pylori* had follicles, thus raising the possibility that lymphoid follicles are the normal response of the gastric mucosa to the *H. pylori* infection. Among their 62 infected patients, eight subjects had lymphoid tissue that virtually obliterated the lamina propria, pushing pits and glands to the side and occasionally creating lymphoepithelial lesions, and the authors labelled these patients as 'hyper-reactive'—those more likely to develop gastric lymphomas.

Another interesting finding of our study was the presence of infectious agents other than *H. pylori* in the gastric tissue, namely EBV or HHV-6, considered to be a possible cause of persistent lymphoid proliferation even in the labial salivary glands.²³ It is also well known that EBV may be found in gastric carcinoma with marked lymphoid stroma.²⁴ To our knowledge this is the first time that EBV or HHV-6 has been detected in the stomach of patients with SS. It was possible to exclude a carry over effect in at least two patients who were negative in mononuclear blood cells, therefore a true infection of the tissue was a possibility. In our series an association between EBV and *H. pylori* was observed in one patient, while HHV-6 was observed in two samples positive for *H. pylori*. As no clear association was found between lymphoid follicles and any of the infectious agents we tested, we may hypothesise that perhaps other yet unknown

infectious agents are harboured in the stomach, as has been described for the salivary glands,²⁵ or that environmental factors²⁶ or auto-antigens²⁷ are involved.

The third main finding of our study was that a clonal expansion, as detected by a sensitive PCR technique, is a fairly common event in SS gastric lesions: it was demonstrated in 42.8% of cases. Most importantly, B cell clonality may appear in the absence of follicles. This is clearly a point of major interest because it emphasises the part played by the environment. Only 55.5% (five of nine) of the cases may be explained by a persistent antigenic stimuli as shown here through histochemical and genotypic analysis of infectious agents. This finding may support those of Pablos *et al*,²⁸ who demonstrated clonally expanded lymphocytes in all minor salivary glands of their 14 patients, yet only one patient developed a plasma cell tumour that differed from the expanded clone in the labial salivary glands. The possibility that B cell clonality may not always be linked to an identifiable infectious agent in SS is reinforced by the results of the second assessment which showed first, that the clonal B cell expansion could occur without any *H. pylori* infection; second, that even after eradication of the infection the clonality was still present, and third, and perhaps more important, that the clonal expansion may appear and vanish over time. Again, this point strengthens the role of the tissue microenvironment in the genesis of MALT and of clonality in these patients.

In summary, we have demonstrated the presence of MALT in the stomach of patients with SS. Even in the most recent review,²⁹ the issue of gastric MALT and of the possible roles of infectious or non-infectious agents in the development of clonal B expansion was neither considered nor discussed. Our data show that a clonal rearrangement may be demonstrated in gastric tissues in which evidence of an infectious agent is not always present. As clonality seems to appear and disappear in the tissue in a fashion apparently independent of infectious antigenic stimuli or treatments, it is difficult to define its prognostic implications. It might represent the first step towards a neoplastic lymphoproliferative disease, which obviously would require the occurrence of additional genetic events, or it might represent the final step of an antigen driven (infectious antigen or autoantigen) lymphoid proliferation.^{27, 30}

It is well recognised that patients with SS are at high risk for lymphoma,^{31, 32} yet gastric lymphoma is not frequent in these patients. Our data strongly support this background literature by providing evidence that *H. pylori* infection is not more frequent among patients with SS than in controls, and that clonal expansion and *H. pylori* infection³³ are not always associated events in this disease. Considering that gastric lymphoma is rare, while clonal B cell expansion appears frequently in gastric tissues of patients with SS, we are led to conclude that these gastric clonalities are 'benign' if pathological features of suspect gastric lymphoma are absent and a lymphoproliferative disorder is not detected elsewhere.

Nevertheless, caution in the use of immuno-suppressive drugs is clearly advisable, as any oncogenic risk might be detrimental to these patients.

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